UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

SUBJECT: Ecological Risk Assessment for the Section 3 registration of the microbial pesticide end-use product ZAP mosquito larvae: PC Code: 069035; EPA File Symbol 89668-U; Decision No. 513757; Submission Nos. 980717, 985153; DP Barcode No: 432413, 433706. MRID Nos: 49530604, 49830704-06

FROM: Gail Tomimatsu, Ph.D. Plant Pathologist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511P

THRU: Shannon Borges, Senior Scientist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division, 7511P

Chris Wozniak, Ph.D., Biotechnology Specialist
Biopesticides and Pollution Prevention Division, 7511P

TO: Seiichi Murasaki, Regulatory Action Leader
Jeannine Kausch, Acting Team Leader
Biopesticides and Pollution Prevention Division, 7511P

A. Background and Regulatory History

MosquitoMate, Inc. submitted an application for a FIFRA Section 3 registration to manufacture and sell an end-use product, "ZAP Males" (EPA File Symbol 89668-U), consisting of male *Aedes albopictus* mosquitoes infected with a strain (referred to as "ZAP") of *Wolbachia pipientis* (note: "ZAP" also refers to the strain of mosquito). The *Ae. albopictus* male mosquitoes in ZAP Males have been artificially infected (manufactured by embryonic microinjection, or "transfection" [Xi, et al., 2006]) with a strain of *Wolbachia pipientis* from *Culex pipiens* mosquitoes. This product is intended to control naturally-occurring populations of *Aedes albopictus*, an invasive and aggressive biting pest with critical public health implications. This species of mosquito is commonly known as the Asian tiger or forest day mosquito and vectors several human viruses, including the West Nile, chikungunya, equine encephalitis, dengue and yellow fever viruses, and possibly the Zika virus. Control of the target mosquito pest population takes place through cytoplasmic incompatibility upon mating, which reduces subsequent egg

hatch after the transfected males mate with their respective wild-type *Ae. albopictus* females. The mosquitoes in ZAP Males are proposed for released at a rate of approximately 1,000 male mosquitoes per acre at the release site.

In 2013 the Agency approved experimental use of the ZAP strain of male *Aedes albopictus* mosquitoes infected with the ZAP strain of *Wolbachia pipientis* in the states of Kentucky, California, New York and Florida. In 2014, the EUP was amended to add one additional test site in California and to amend the expiration date from July 29, 2015 to October 31, 2015 for testing in Kentucky, California and Florida; and from July 29, 2016 to September 30, 2016 for testing in New York. The Agency also raised the issue of the potential for horizontal transmission of the endosymbiotic bacterium to other aquatic and terrestrial invertebrates and requested additional data and information to clarify potential adverse effects to nontarget organism populations (insects and invertebrates) for larger scale releases and a potential Section 3 registration [U.S.EPA, 2013]. In 2015, the Agency approved an Experimental Use permit to release male *Aedes aegypti* WB1 mosquitoes infected with *Wolbachia pipientis* wAlbB strain; this particular strain of *Wolbachia pipientis* was isolated from wild-type *Aedes albopictus* within the U.S. *Aedes aegypti* is another nuisance mosquito critical to public health, as it vectors dengue, chikungunya, Zika, and yellow fever viruses in the United States and its territories.

One of the most critical considerations for evaluating potential adverse effects to nontarget organisms (including humans) involves the possible horizontal or lateral transmission of a viable microbial active ingredient from one host species to another (host) species. As discussed below in the section, Nontarget Insects and Honeybees, the Agency anticipates no significant adverse effects from horizontal transmission of *Wolbachia pipientis* infections from mosquitos (*Ae. albopictus* or *Ae. aegypti*) that have been transfected with the ZAP or wAlbB strains. Notably, horizontal transmission of a prokaryote to a eukaryote may or may not involve exchange of genetic elements. Exchange of genetic elements between phylogenetically distant and reproductively isolated species is commonly referred to as horizontal gene or lateral gene transfer (HGT or LGT) [Hou, et al., 2014; Dunning Hotopp, et al., 2007], and is considered extremely rare. Because of the intimate symbiotic relationships that *Wolbachia* spp have with their respective eukaryotic invertebrate hosts, HGT or LGT is possible, albeit on an evolutionary time scale, since horizontal transmission of the *Wolbachia* bacteria is thought to occur on a similar time scale [Hou, et al., 2014; Klasson, et al., 2009; Dunning Hotopp, et al., 2007; Woolfit, et al., 2009].

B. Ecological Effects Data

MosquitoMate, Inc. submitted scientific rationale to fulfill nontarget organism data requirements for a full Section 3 registration for a microbial pesticide. The applicant also conducted a study to support a conclusion of no interspecific horizontal transmission of *Wolbachia pipientis* through an oral route of exposure. A summary of these data are provided in Table 1 and within the risk assessment below, and full review of the rationale is contained within the attached Data Evaluation Records (DERs). Some of these data were previously reviewed to support the previous experimental use permits for *Wolbachia pipientis* ZAP strain [U.S. EPA 2014; U.S. EPA, 2013] and for similar testing with another strain of *Wolbachia* (*W. pipientis* strain wAlbB from *Ae. albopictus* mosquitos) in *Ae. aegypti* males [U.S. EPA, 2015]. These data were

acceptable for the 2015 EUP due to its similarity in scope and intent and lack of concerns for requiring new or additional information [U.S. EPA, 2015].

Wolbachia pipientis ZAP strain is a new a.i., and thus requires a review to assess the potential risks to nontarget organisms that may result from its use. This memorandum contains BPPD's ecological risk assessment for the pending federal registration of the *Wolbachia pipientis* ZAP strain. The ecological risk assessment also applies to assessing potential risks to nontarget organisms for the experimental testing of another strain, *W. pipientis* wAlbB.

Table 1. Status of data submitted to comply with nontarget organism data requirements published in 40 CFR § 158.2150.

Data Requirement	**OPPTS Guideline No.**	**Results Summary and Classification**	**MRID No.**
• Avian oral toxicity • Freshwater fish toxicity/pathogenicity • Nontarget plant testing • Honey bee testing • Freshwater invertebrate toxicity/pathogenicity • Nontarget insect testing	885.4050 885.4200 885.4300 885.4380 885.4240 885.4340	Rationale submitted in 2013 [U.S. EPA, 2013] and in the present application provides sufficient information to determine that toxicity/pathogenicity by ZAP mosquito larvae (transfected by *Wolbachia pipientis* ZAP strain) to nontarget organisms is not expected for this use, based on the lack of an infective route of exposure. *Wolbachia* have never been detected in birds, mammals, fish, reptiles or plants [Popovich, et al 2010], and are restricted to several insect classes, some Crustaceae and Nematoda [Werren et al., 2008; Hilgoenboecker et al., 2008]. **Classification: Acceptable.** Rationale was acceptable for the EUP and have been determined acceptable for the Section 3. Requested data and information regarding potential for horizontal transmission were provided.	49010119 49010120 49010121 49010122 49010123 49010124 49530604

Data Requirement	OPPTS Guideline No.	Results Summary and Classification	MRID No.
• Avian inhalation toxicity/pathogenicity •Wild Mammal toxicity/pathogenicity • Estaurine and Marine Organism toxicity	885.4100 885.4150 885.4280	Submitted rationale in the present (2016) application provides sufficient information to determine that toxicity/pathogenicity by ZAP mosquito larvae (transfected by *Wolbachia pipientis* ZAP strain) to these nontarget organisms is not expected for this use, based on the lack of an infective route of exposure. *Wolbachia* have never been detected in birds, mammals, fish, reptiles or plants [Popovich, et al 2010], and are restricted to several insect classes, some Crustaceae and Nematoda [Werren et al., 2008; Hilgoenboecker, et al., 2008]. **Classification: Acceptable.** Rationale was determined acceptable for the Section 3. Requested data and information regarding potential for horizontal transmission were provided.	49830704 49830705 49830706 49530604
Freshwater invertebrate toxicity/pathogenicity (*Corethrella appendiculata*)	885.4240	Results of a laboratory study testing the potential for horizontal transmission of *Wolbachia pipientis* DNA from mosquito larvae (ZAP strain) to the mosquito predatory midge via oral exposure was examined over a period of nine months. **Classification: Unacceptable.** Insufficient reporting of details rendered the study inconclusive.	49530604

C. Ecological Exposure and Risk Characterization

Wolbachia pipientis is a naturally-occurring obligate intracellular bacterium and is capable of infecting invertebrate species, which include insects, crustaceans and the filarial nematodes. These alpha proteobacteria endosymbionts are transmitted maternally and may alter host biology and phenotypes through reproductive manipulations, such as feminization, parthenogenesis, cytoplasmic incompatibility, male killing and sperm–egg incompatibility. They have not been detected in vertebrates, fish or plants; and cannot survive outside their host's cytoplasm.

Manipulation of a host's reproduction system by arresting the development of early embryos is commonly referred as cytoplasmic incompatibility, a method of inducing male sterility ("Incompatible Insect Technique, or IIT), and is the basis for the pesticidal mode of the active ingredient, *Wolbachia pipientis* for the end-use product, ZAP Males. Cytoplasmic incompatibility naturally occurs in other *Wolbachia*- infected mosquitoes, as well as other insect species [Xi, et al., 2006] and provides infected female hosts with a reproductive advantage relative to uninfected females. Cytoplasmic incompatibility results in loss of paternal

chromosomes and embryo death, and is expected to occur only when the male has been infected by a *Wolbachia* type (e.g., clade) that is not present in his mate [Dobson, et al., 2002]. More importantly, ZAP males are self-limiting as 'dead-end' hosts (MRIDs 49530604, 4983070406), because the ZAP males' do not transmit *Wolbachia* nor its genetic material, and their mates do not produce viable offspring. Because this pesticidal point of action is very targeted and unique, it is an attractive complement for mosquito management programs that rely on a number of chemical adulticides (and larvacides) and biopesticidal larvacides. It is especially noteworthy that the presence of *Wolbachia pipientis* infection did not appear to affect the efficacy of chemical pesticides (bifenthrin and temephos) or biopesticides (*Bacillus thuringiensis* subsp. *israeliensis* and *s*-methoprene) in control of *Aedes aegypti* [Endersby and Hoffman, 2012].

Wolbachia's widespread and global distribution in diverse invertebrate hosts has been hypothesized to be consequences of horizontal transmission of the microorganism across species boundaries, presumably resulting from complex, intimate ecological relationships (e.g., host-parasite, predator-specific prey interactions, or endosymbiotic gene transfer) that have occurred on an evolutionary time scale [Hou, et al., 2014; Zug, et al 2012; Werren, et al., 2008; Dunning Hotopp, et al., 2007]. In the U.S., the ZAP and wAlbB strains of *Wolbachia pipiens* occur in the mosquitoes *Culex pipiens* and *Ae. albopictus*, (their respective original host species), therefore all nontarget organisms have a history of exposure to these strains of *Wolbachia pipientis*. Endosymbiotic gene transfer [Hou, et al., 2014] represents the exchange of genetic elements between an endosymbiont such as *Wolbachia* and its host, and will be discussed in greater detail in the section on *Nontarget Insects and Honeybees*.

There are several hundred different mosquito species world-wide. The females of most mosquitoes are zoophilic; although few seem to prefer human blood (anthrophilic) as opposed to other animals. The larvae of a non-biting mosquito, *Toxorhynchites rutilus* prey on other mosquito larvae; adult *Toxorynchites* feed on plant nectar. All mosquitoes are dependent upon water for reproduction and survival; and several species can overlap in geographic distribution, adapting to different types of aquatic habitats. In the U.S., invasive and anthrophilic species *Ae. albopictus* and *Ae. aegypti* are largely found in artificial standing water habitats in urban/suburban areas (e.g., spare tires, ornamental ponds and container gardens), whereas native *Ae. triseriatus* have been largely detected in standing waters (treeholes, small ponds or puddles) in hardwood or heavily forested areas [Lounibos, et al., 2001]. Both *Ae. albopictus* and *Ae. aegypti* are considered non-indigenous to the U.S.; *Ae. albopictus* arrived in the U.S.(i.e., New World) in 1985 in used automobile tires from Japan [Lounibos, et al., 2002], whereas *Ae.aegypti* probably arrived centuries ago from Africa, perhaps as early as 1495 in Haiti [Cloudsley-Thompson, 1976 as cited in Powell and Tabachnick, 2013]. The presence of certain species of mosquito genera *Aedes, Toxorynchites* and other mosquito species were noted in experimental use sites, though no quantitative data were provided [U.S. EPA, 2016].

Despite its relatively recent arrival in the New World *Ae. albopictus* is considered more invasive than its relative, *Ae. aegypti*. *Aedes albopictus* colonies reduced tree-hole populations of *Aedes aegypti* and native *Aedes triseriatus* [Lounibos et al, 2001], and competitively displaced *Aedes triseriatus* in temperate containers [Rochlin et al., 2013]; although *Ae. triseriatus* was not reduced to the point of extinction in the 2013 study. Whereas higher winter temperatures and precipitation favored increased *Ae. albopictus* abundance in the study by Rochlin et al [Rochlin,

et al., 2013], eggs of *Ae. albopictus* also hatched more rapidly than those of *Ae. triseriatus* in tree hole studies with the predatory mosquito *Toxorynchites rutilus* (Lounibos, et al., 2002). For both of these studies, the native *Ae. triseriatus* were able to re-establish their populations fairly easily, even within areas where invasive species had temporarily displaced them. The estimated distributions and invasiveness of *Ae. albopictus* and *Ae. aegypti* populations overlap, however *Ae. albopictus* generally extends more north and northeast than does *Ae. aegypti* [Centers for Disease Control, 2016].

It is important to note for the risk assessment that the ZAP strain of *Wolbachia* was not found in the *Ae. albopictus* mosquitoes at the experimental sites utilized in the EUP, so there is no known history of exposure of nontarget organisms to this species of mosquito with this strain of *Wolbachia*. However, the ZAP strain occurs in *Culex pipiens* in the U.S; *C. pipiens* is the original host species. The wAlbB strain involved in the 2015 EUP is present in *Ae. albopictus* within the U.S. Therefore, exposure of nontarget organisms to the *Wolbachia pipientis* in ZAP males occurs within the U.S., as does exposure to the *W. pipientis* wAlbB strain.

1. Terrestrial Animals and Plants

Birds and Mammals

Birds and wild mammals will be exposed to *Wolbachia pipientis* ZAP strain primarily through ingestion of infected arthropod prey, or in extremely rare instances, from zoophilic female mosquitoes that may be missed during the screening of the end-use product, as males are segregated from females by sizing. The predicted rate of 1 female release per 250,000 males released was confirmed at a power of 0.95; and the 0.95 power assumes 95% of the release events will be at the 1 per 250,000 rate [(MRID No.49830702); U.S. EPA. 2016]. Because of the low expectation that females would be released in the end-use product ZAP Males, the only reasonable route for bird and wild mammal acute exposures to *W. pipientis* ZAP strain would be through an oral route. As discussed in the ecological risk assessment for the EUP [U.S. EPA, 2014] and in the open literature, *W. pipientis* is not a recognized pathogen of birds, nor does it routinely infect vertebrates [Werren, et al 2008]. Furthermore, the obligate endosymbiont *W. pipientis* cannot survive or persist in a vertebrate organism without its invertebrate host (MRIDs 49530604, 49830704-49830706), and thus there is currently no reason to suspect that pathways to infectivity and pathogenicity exist in these nontarget organisms. Also, in two immunological tests, humans repeatedly bitten by *W. pipientis*-infected mosquitoes did not develop an antibody against *W. pipientis*, which indicates that *W. pipientis* is not transferred via mosquito saliva [Popovici et al., 2010]. Therefore, adverse effects are not expected in birds or wild mammals as a result of release of male mosquitoes infected with *W. pipientis* ZAP strain.

Nontarget Insects and Honeybees

Nontarget insects may be exposed to *W. pipientis* ZAP strain through consumption of the *W. pipientis* ZAP male mosquitoes or through some undetermined (or as yet unreported) route of interspecific horizontal transmission of the MPCA from living mosquitoes. There was no evidence of horizontal transmission of *W. pipientis* from ZAP Male mosquitoes to the mosquito predatory midge (*Corethrella appendiculata*) via a predator-prey interaction in a laboratory

study (MRID 49530604); additional details are provided below in the nontarget aquatic organisms section. The applicant also cited evidence that there is no apparent route of horizontal transmission of *Wolbachia* through spider predator-prey relationships [Cordaux et al., 2001; Yun, et al., 2011], and that horizontal transmission may only be accomplished via specific transfers under precise laboratory methods, i.e., through afore-mentioned embryonic microinjection techniques [Xi, et al., 2006; MRID 49530604], used for transfection of ZAP *Ae. albopictus*. The results of the cited works suggest that if interspecific horizontal transmission of *Wolbachia pipiens* ZAP strain were to occur, it would happen on an evolutionary scale under natural conditions, however the mechanism(s) by which this could be accomplished is not yet clear. Other predaceous insects include the elephant mosquito (*Toxorhynchites rutilus*) and dragonflies (Order Odonata), which may have other strains of *Wolbachia pipientis*. *Wolbachia pipientis* is the type species and is among the most abundant intracellular bacteria genus discovered thus far, as it has been estimated that >65% of insect species harbor *Wolbachia,* infecting at least one million insect species alone [Werren, et al., 2008].

Horizontal transmission between insect genera or species is presumably rare in a predator-prey relationship as noted in the previous paragraph, however could be more frequent and not as rare, especially in intimate associations such as, insect parasitoids-insect host relationships [Vavre, et al., 1999; Heath et al., 1999; Pattabhiramaiah, et al 2011] or, close interactions involving shared food sources [Evison, et al. 2012]. Potential natural routes of horizontal transmission were demonstrated in several laboratory studies involving *Drosophila*-hymenopteran parasitoid interactions [Vavre et al., 1999; Heath et al., 1999], and in the host-parasite interaction of honeybees (*Apis mellifera carnica*) and the ectoparasitic mite, *Varroa destructor* [Pattabhiramaiah et al 2011]. Vertical transmission of the transfected *Wolbachia* was noted in subsequent generations of respective offspring [Heath, et al 1999; Pattabhiramaiah et al 2011], although no observations with respect to adverse effects in reproductive or fitness capacity in subsequent generations were reported. The Agency is not aware of insect parasitoids of mosquitoes that would have the necessary host-parasite relationship for cross-infection by *Wolbachia pipientis* ZAP strain to other nontarget insects, including predaceous mosquitoes.

Close interactions involving shared food sources such as pools of nectar in flowering plants [Evison, 2012] by multiple pollinators, such as honeybees or nontarget mosquitoes (e.g., *Toxorhynchites rutilus*) may offer additional exposure scenarios where *Wolbachia* may be vectored or picked up by these visiting consumers. Although such chance interactions are probably unlikely, since most research shows that *Wolbachia* needs a living host and predominantly reside in the host's reproductive tissues, there are reports where *Wolbachia* can briefly exist outside of host cells. For example, in one report, *Wolbachia* was shown to persist long after the host cells had died in tissue culture experiments [Fallon, 2008 as cited in Werren et al., 2008]. Results of another study indicated that *Wolbachia* can enter the ovary of *Drosophila melanogaster* when they are injected into the adult body, traverse cell membranes and eventually enter developing eggs [Frydman, et al., 2006, as cited in Werren, et al., 2008]. *Wolbachia* must be present in mature and viable eggs of the new host for dissemination in the new population [Heath, et al 1999], as in the case of ZAP Males (MRID 49680201). Release of ZAP Males is not anticipated to significantly increase the amount of naturally-occurring *Wolbachia pipientis*, as they are a dead-end hosts, and the bacterial symbiont was determined to be absent in the

mosquito saliva [Popovici, 2010]. Therefore, adverse effects are not expected in nontarget insects or honeybees as a result of release of male mosquitoes infected with *W. pipientis* ZAP.

There was no evidence of unintentional ZAP female release nor of horizontal transmission of *W. pipientis* ZAP infection from released ZAP males (MRIDs 49530601 and 49680201) in experiments conducted under the EUP. In 2013 and 2014, eggs were recovered from oviposition traps that were placed at the ZAP male release site and were hatched/reared in the laboratory. Immature mosquitoes were reared to adults and tested for the *w*Pip infection using PCR. All field collected individuals showed the *Wolbachia* infection type that is naturally present in *Ae. albopictus*. The inability to detect *w*Pip infection in these collected individuals (a total of 32 and 17 individuals collected in 2014 and 2013 respectively) supports the hypotheses that there was no horizontal *w*Pip transmission or accidental release of ZAP females. Additional detail is available in the DERs for these efficacy studies. The Agency considered horizontal transmission to be infrequent and did not anticipate adverse effects to nontarget insects and honeybees for the duration of the EUP [U.S. EPA, 2014; U.S. EPA, 2013].

As stated previously, horizontal gene or lateral gene transfer (HGT or LGT) involves the exchange of genetic elements between phylogenetically distant and reproductively isolated species. Presently, it is uncertain as to the extent that HGT has in interspecific horizontal transmission of *Wolbachia pipientis* from one insect species to another. Until recently, HGT [Hou, et al., 2014; Dunning Hotopp, et al., 2007] was traditionally thought to be unlikely between prokaryotes and eukaryotes. Although intensive laboratory studies revealed that genetic fragments, ranging in size from single genes to even the entire genome, have been horizontally transferred from *Wolbachia* to their insect hosts [Dunning Hotopp, et al., 2007], such genes may have been discovered during tetracycline treatment [e.g., Kondo, et al., 2002], or transcription levels were low or potentially non-functional [Woolfit, et al., 2009; Dunning Hotopp, et al., 2007], or they may lose their evolutionary significance after possible degradation or loss [Klasson, et al., 2009]. Conversely, HGT may occur more frequently [Werren, et al., 2008]; as HGT could be bi-directional (from eukaryotic host to prokaryotic endosymbiont, and vice-versa, "host↔ endosymbiont") in prolonged endosymbiotic relationships, such as the obligate mutualistic association of *Wolbachia* with its filarial nematode host, *Onchocerca ochengi* [Fenn, et al., 2006]; or where host genomes may be occasional, but important sources of novel genetic material for *Wolbachia pipientis*, and impacting endosymbiotic relationships and evolution [Woolfit, et al., 2009].

Potential adverse effects to nontarget invertebrates as a result of HGT in interspecific horizontal transmission of the ZAP strain of *W. pipientis* in its respective host, *Ae. albopictus*, would need further intensive research that involves controlled releases of these artificially-infected mosquitocides. While the Agency anticipates no adverse effects to populations of *Ae. albopictus*; over time, such effects could diminish or change, and result in other phenotypic symbioses between *Wolbachia* and these mosquito species, and perhaps other non-mosquito hosts [e.g., see Woolfit, et al., 2009]. This would also apply to the wAlbB strain in *Ae. Aegyptii*.

Based on information from peer-reviewed scientific literature as discussed above, HGT involving prokaryotes and eukaryotes is somewhat controversial, as it involves demonstration of novel functionality, i.e.: (1) the donor DNA has to be delivered into the recipient cell, (2) the

target sequences must integrate into the host's genome, and (3) demonstration that these genes could be expressed in host organisms or cells [Ochman, et al., 2000: as cited in Hou, et al., 2014]. Furthermore, longevity and integration of the transferred genes into the biology of the recipient taxon are necessary to fulfill the basic criteria for evolutionary significance [Blaxter, 2007: as cited in Woolfit, et al., 2009]. Further research is needed to determine whether HGT has a role in interspecific horizontal transmission of *Wolbachia*, particularly whether or not such transfers result in expressible and functional proteins. Because of the complicated and speculative nature surrounding these endosymbionts, these effects are likely to be transient or minimal over time.

Therefore, the Agency does not anticipate adverse effects to nontarget insects and honeybees as a consequence of release of male mosquitoes infected with *W. pipientis* ZAP strain.

Nontarget Plants

As discussed in the ecological risk assessment for the EUP [U.S.EPA, 2013], *Wolbachia* species have not been reported as plant pathogens, and there is no reason to believe that *Wolbachia* is pathogenic to plants (includes aquatic plant taxa). Adverse effects are not expected in nontarget plants as a result of release of male mosquitoes infected with *W. pipientis* ZAP strain.

2. Aquatic Animals and Plants

Freshwater, Marine/Estuarine Fish and Invertebrates and Aquatic Plants

Nontarget fish and aquatic invertebrates would have to be exposed to *W. pipientis* ZAP strain through consumption of the *W. pipientis* ZAP strain bacteria. Since ZAP male mosquitoes are not expected to produce viable offspring, larvae containing the *W. pipientis* ZAP strain bacteria are not expected to be present in the water. The only conceivable way they may be exposed would be through some undetermined (or as yet unreported) route of interspecific horizontal transmission from living mosquitoes. The applicant was asked to clarify whether or not horizontal transmission was possible to other invertebrates that might encounter *Wolbachia pipientis* ZAP strain [U.S.EPA, 2013].

Results of a submitted laboratory study showed no evidence of horizontal transmission of *W. pipientis* from ZAP male mosquitoes to the mosquito predatory midge (*Corethrella appendiculata*) via a predator-prey interaction (MRID 49530604). Groups of the larval stage of the test animal were fed ZAP Males over a period of nine months in 2013 through 2014. Testing for presence of *W. pipientis* DNA was confirmed by PCR amplification in the adult after pupal emergence. In the fourteen cohorts tested, there was no evidence of *Wolbachia pipientis* ZAP strain DNA after ingestion of the *W .pipientis* ZAP larvae, suggesting that interspecific horizontal transmission does not occur via a prey-predator relationship. This study was lacking in many details, including lack of inclusion of negative controls, lack of reporting on positive controls, and uncertainty regarding sampling. Therefore, the study results were determined to be unreliable. This study was not requested or required by EPA, and was submitted to support a conclusion that horizontal transmission would be unlikely in a predator-prey interaction. Nonetheless, while uncertainty regarding the above conclusion is low, the study would be useful

to further reduce any remaining uncertainty. It is recommended that EPA request additional details regarding the exposure and subsequent demonstration of the lack of horizontal transmission in the submitted study. Alternatively, EPA recommends a repeat of the study with more attention to reporting details of design and results.

In the ecological risk assessment for the EUP [U.S. EPA, 2014] and in the open literature, *W. pipientis* is not a recognized pathogen of fish, nor do they routinely infect vertebrates (Werren, et al 2008). On the basis of this information, evidence from the open literature indicating that horizontal transmission is likely very rare, and results of the efficacy studies showing no horizontal transmission of *Wolbachia* from ZAP males following release and submitted rationale, the Agency does not anticipate immediate adverse effects to nontarget organisms.

E. Ecological Risk Conclusions

EPA has determined that no adverse effects are anticipated for nontarget organisms as a result of this registration for ZAP Males, which contains male *Ae. albopictus* that have been transfected with *Wolbachia pipientis* ZAP strain. In addition, no adverse effects are anticipated for nontarget organisms for the experimental permit to release *Ae. aegyptii* transfected with *W. pipientis* wAlbB strain [U.S. EPA, 2015]. Therefore, since adverse effects are not anticipated to non-target organisms, a "No Effect" determination is also made for direct and indirect effects to federally listed endangered and threatened species, and for their designated critical habitats.

SELECTED REFERENCES

Centers for Disease Control (CDC). 2016. http://www.cdc.gov/zika/pdfs/zika-mosquito-maps.pdf , April.

Dunning Hotopp, J.C., M. Clark, D.Oliveira, and 17 additional co-authors, including J.Werren.2007. Widespread lateral gene transfer from intracellular bacteria to multicellular eukaryotes. Science. 317:1753-1756.

Endersby, N.M. and A. A. Hoffmann. 2012. Effect of Wolbachia on insecticide susceptibility in lines of *Aedes aegypti*. Bulletin of Entomological Research pp 1-9. Doi:10.1017/S0007485312000673

Fenn, K., C. Conlon, M. Jones and 4 additional co-authors, including M. Blaxter. 2006. Phylogenetic relationships of the *Wolbachia* of nematodes and arthropods. PLoS Pathogens 2(9):e94. DOI:10.1371/journal.ppat.0020094.

Evison, S., K.Roberts, L. Laurenson, S. Pietravalle, J. Hui, J. Biesmeijer, J.Smith, G. Budge, and W. Hughes. 2012. Pervasiveness of parasites in pollinators. PLoS One 7:e30641. http://www.plosone.org

Heath, B., R.Butcher,W.Whitfield and S.Hubbard.1999.Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism. Current Biology 9:313-316. Brief Communication.

Hilgoenboecker, K., P. Hammerstein, P. Schlattmann, A. Telschow and J.Werren.2008.How many species are infected with *Wolbachia*?—a statistical analysis of current data.FEMS Microbiology Letters 281:215-230.

Hou, Q. J.He, Y.Ye, and 6 additional co-authors, including C. Zhu. 2014. A case of horizontal gene transfer from *Wolbachia* to *Aedes albopictus* C6/36 cell line. Mobile Genetic Elements 4:e28914; http://dx.doi.org/10.416/mge.28914.

Klasson, L., Z. Kambris, P.E. Cook, T. Walker and S. Sinkins. 2009. Horizontal gene transfer between *Wolbachia* and the mosquito *Aedes aegypti*. BMC Genomics 10:33. Doi:10.1186/1471-2164/10/33; http://www.biomedcentral.com/1471-2164/10/33.

Kondo, N. N. Nikoh, N. Ijichi, M. Shimada and T. Fukatsu. 2002. Genome fragment of Wolbachia endosymbiont transferred to X chromosome of host insect. PNAS 99:14280-14285.

Lounibos, L., G. O'Meara, R. Escher and 5 co-authors including S. Juliano. 2001. Testing predictions of displacement of native *Aedes* by the invasive Asian tiger mosquito *Aedes albopictus* in Florida, USA. Biological Invasions 3:151-166.
Powell, J. and W. Tabachnick. 2013. History of domestication and spread of *Aedes aegypti* – A review. Mem. Inst. Oswaldo Cruz, Rio de Janeiro. 108 (Suppl.1): 11-17. Doi:10:1590/0074-0276130395.

Popovici, J., L. Moreira, A. Poinsignon,I. Iturbe-Ormaetxe, D. McNaughton, S. O'Neill.2010.Assessing key safety concerns of a *Wolbachia*-based strategy to control dengue transmission by Aedes mosquitoes. Memorias do Instituo Oswaldo Cruz, Rio de Janeiro, 105:957-964.

Pattabhiramaiah, M., D. Brückner, M. Reddy.2011.Horizontal transmission of *Wolbachia* in the honeybee subspecies *Apis mellifera carnica* and its ectoparasite *Varroa destructor*. International J. of Environmental Sci. 2:526-535.

Rochlin, I., R. Gaugler, E. Williges and A. Farajollahi. 2013. The rise of the invasives and decline of the natives: insights revealed from adult populations of container-inhabiting *Aedes* mosquitoes (Diptera: Culicidae) in temperate North America. Biological Invasions 15:991-1003. DOI 10.1007/s10530-012-0345-3

U.S. EPA. 2013. Environmental Risk of *Wolbachia pipientis* ZAP strain (EPA Reg. No. 89668-EUP-1); PC Code 069035, Decision No. 484885, DP Barcode No. 417109.

U.S. EPA. 2014. Environmental Risk Assessment for an Amendment to the Experimental Use Permit for *Wolbachia pipientis* ZAP strain (EPA Reg. No. 89668-EUP-1); PC Code 069035, Decision No. 484885, DP Barcode No. 417109.

U.S. EPA. 2015. Environmental Risk Assessment of *Wolbachia pipientis* wAlbB strain for an Experimental Use Permit for Release within Infected *Aedes aegypti* WB1 strain Mosquitoes in

California (EPA File Symbol 88877-EUP-E); Decision No. 500458, DP Barcode Nos. 426682; MRID 49579406; PC Code 069039

U.S. EPA.2016. Review of Efficacy data on male releases of *Ae. albopictus* infected with ZAP strain *Wolbachia pipientis* and BPPD modeling analyses for population dynamics of *Ae. albopictus* (EPA File Symbol 89668-U); Decision No. ; DP Barcode No. ; MRIDs 49680201, 49783101 and 49830703; PC Code 069035

U.S. EPA.2016. Review of Product Characterization, Manufacturing Process, and Human Health Data for the Section 3 Registration of the ZAP strain *Wolbachia pipientis* in *Ae. albopictus*. (EPA File Symbol 89668-U); Decision No. ; DP Barcode No. ;

Vavre,F., F.Fleury, D.Lepetit, P.Fouileet and M.Boulétreau.1999.Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations.Molecular Biol. Evol. 16:1711-1723.

Werren, J. H., L. Baldo and M. Clark. 2008. *Wolbachia*: masters of invertebrate biology. Nature Reviews/Microbiology.6: 741-751.

Woolfit, M., I. Iturbe-Ormaetxe, E. McGraw and S. O'Neill. 2009. An ancient horizontal gene transfer between mosquito and the endosymbiotic bacterium Wolbachia pipientis. Mol. Biol. Evol. 26(2):367-374. doi:10.1093/molbev/msn253.

Xi, Z., C.Khoo and S.L.Dobson.2006.Interspecific transfer of *Wolbachia* into the mosquito disease vector *Aedes albopictus*. Proc. Royal Society B. 273:1317-1322.